HYDROGENASE ACTIVITY ASSAYS ON *METHYLOCOCCUS CAPSULATUS* (BATH)

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Abstract

The presence and activity of hydrogenase in the thermotolerant methanotroph *Methylococcus capsulatus* (BATH) was demonstrated. Hydrogenase activity was found by two independent enzyme assays in cells cultivated under routine methanotroph-growing conditions. Hydrogenase activity without prior induction by molecular hydrogen or nitrogen fixing growth suggests that a constitutively expressed hydrogenase is present in *Mc. capsulatus* (BATH). Hydrogen utilising activity reached half of the maximal value at 2% hydrogen in the headspace. Molecular hydrogen was suitable to provide reducing power for the sMMO in TCE degradation via the hydrogenase.

Key words: Biotechnology, Hydrogenase, Methane monooxygenase, Biohydrogen, Methanotrophic bacteria, Thermotolerance, *Methyloccoccus capsulatus* (Bath).

Introduction

Methane oxidising bacteria (methanotrophs) have attracted considerable interest over the past twenty years due to their potential in producing bulk chemicals, such as propylene oxide, single cell protein and for use in biotransformation (DALTON et al., 1995). More recently, their ability to degrade the groundwater pollutant trichloroethylene (TCE) and other chlorinated compounds has also been examined (OLDENHUIS and JANNSEN, 1993). Methanotrophs are unique in that they only grow on the one-carbon compound, methane. Some will also grow on methanol. They cannot use heterotrophic/multicarbon compounds as sole carbon and energy

sources. Methanotrophs are also an important group of microorganisms as they are a major sink for biologically produced methane in the biosphere and appear to be ubiquitous in nature. They have been isolated from freshwater and marine environments, soils and sediments (BOWMAN et al., 1993) and a number of different genera and species now exist in culture. Despite the widespread nature of methanotrophs in the environment and their biotechnological potential, the thermophilic isolates (> 50°C) have received surprisingly little attention.

Methane is oxidised by methanotrophs using the enzyme methane monooxygenase (MMO). Further knowledge of this enzyme will aid the design of catalysts and development of biocatalysts crucial for the effective use of methane as a fuel and industrial feedstock. MMO exists in two forms in the cell, depending on the availability of copper in the environment (STANLEY et al., 1983). The soluble enzyme complex (sMMO) is present in some but not all methanotrophs. It has been extensively studied in *Methylococcus capsulatus* (BATH), *Methylosinus trichosporium* OB3b and *Methylocystis* strain M (DALTON, 1992; LIPSCOMB, 1994; NAKAJIMA et al., 1992). The sMMO is very unusual in that it will also cooxidise a wide variety of aliphatic, aromatic and halogenated hydrocarbons (COLBY et al., 1977), making it an extremely versatile enzyme for biocatalysis and biodegradation processes. *Methylocystis* strain M is being extensively used in Japan for bioremediation of TCE contaminated groundwater and for biodegradation of TCE (OKADA et al., 1995). The genes encoding the sMMO from *Ms. trichosporium, Mc. capsulatus* and *Methylocystis* strain M have been cloned, sequenced and extensively characterised at the molecular level (MURRELL, 1992, 1994; MCDONALD et al., 1997).

The other form of MMO, found in all methanotrophs, is the membrane bound or particulate form (pMMO). This has proved extremely difficult to purify in active form (NGUYEN et al., 1996). It consists of at least two membrane associated polypeptides which have recently been solubilised in an active state (SHIEMKE et al., 1995). It has a narrower substrate specificity than sMMO does, but has been shown to oxidise trichloroethylene (TCE) albeit at lower levels than sMMO (DISPIRITO et al., 1992). The genes encoding this enzyme have also been cloned and are currently being studied at the molecular level (SEMRAU et al., 1995; HOLMES et al., 1995).

Both MMO enzymes require reducing equivalents for their catalytic activity. Under physiological conditions this is supplied by the oxidation of the methanol produced. Since biodegradation processes such as the decomposition of chlorinated aliphatic compounds by MMO are cooxidation processes, alternative ways of supplying reducing power are needed. The cleanest and economically most promising alternative is the use of hydrogen.

Hydrogenases are metalloenzymes that catalyse the reversible oxidation of molecular hydrogen and, as such, are important enzymes in anaerobic metabolism of both chemotrophic and phototrophic bacteria (VIGNAIS et al., 1995). Because the

enzyme is involved in producing hydrogen from water, nitrogen fixation, biogas production, corrosive sulphate reduction, and specific hydrogenation reactions, it has generated considerable interest as a biological catalyst involved in reactions of major commercial importance.

All hydrogenases contain FeS clusters of various types, but only a small group of enzymes containing only FeS clusters have been characterised. The majority of known hydrogenases have Ni, and a few enzymes contain Ni and Se, in addition to the FeS redox clusters.

Very little is known about hydrogenases in methanotrophs. DEBONT (1976) reported hydrogen uptake activity in *Methylosinus* strain 41. This activity was induced by nitrogen fixing growth conditions only. The presence of an uptake hydrogenase was concluded from the fact that acetylene reduction by whole cells could be driven by molecular hydrogen. Constitutive hydrogen evolving activities from formate under anaerobic conditions were reported for *Methylomicrobium album* BG8 and *Ms. trichosporium* OB3b (KAWAMURA et al., 1983). Maximum activities were 1.5 and 0.45 nmoles hydrogen formed/min x mg dry cell for *Mm. album* BG8 and *Ms. trichosporium* OB3b, respectively. TAKEDA (1988) showed that *Methylocystis* T-1 produced hydrogen under nitrogen fixing growth conditions in the presence of 1.5-5.0% O_2 in the headspace. Nitrogen fixation was inhibited at higher oxygen concentrations, whereas no hydrogen was detected when the ratio of oxygen was decreased below 1.5%. These results suggested the presence of an uptake hydrogenase sensitive to oxygen concentrations exceeding 1.5%. CHEN and YOCH (1987) reported distinct constitutive and inducible hydrogen uptake activities in *Ms. trichosporium* OB3b. The constitutive activity was observed under all growth conditions tested and had a v_{max}=60 nmoles hydrogen consumed/min x mg dry cell, and a very high K_M of 50% for H_2. Induction of the inducible activity could be achieved in mature cells by overnight incubation under an atmosphere of 50% hydrogen, 5% air and 45% argon in the absence of methane and ammonia. The inducible activity had a v_{max}=32 nmoles hydrogen consumed/min x mg dry cell, and a distinctly lower K_M of 1% for H_2. The hydrogen uptake activity in *Ms. trichosporium* OB3b was shown to be able to supply reducing power for both sMMO and pMMO activities (SHAH et al., 1995).

MATERIAL AND METHODS

Cultivation of organisms

Unless otherwise indicated, strains were grown in NMS medium (WHITTENBURY and DALTON, 1981) containing 0. 4 μM $CuSO_4$, 7. 5 μM $NiCl_2$ and 16 μM $NaMoO_4$. VCR NMS medium consisted of the above medium supplemented with vitamins (KANAGAWA et al., 1982). To solidify media, 1. 5 % (w/v) of Bacto agar (Difco Laboratories) was routinely added. Liquid cultures were grown in 15 or 100 ml of medium in 50 or 500 ml conical flasks shaken at 200 rpm. Flasks were stoppered with rubber

Suba Seals. Head space was filled with a methane:air:CO_2=50:48:2 gas mixture. The gas-to-liquid ratio in the bottles and flasks was 4:1. Incubation temperature was 43°C. Liquid cultures were also grown in a „BioFlo IIC" New Brunswick fermenter equipped with 1.5 l glass vessel. Operational conditions of the fermenter were 40°C, 150–250 rpm agitation, pH 6.8, continuous addition of methane and air at 75 and 50 ml flow rates, respectively. Cell density was kept between OD_{540}=1.0–2.0 via continuous fermentation. Plates were incubated in anaerobic jars under the same methane-air-CO_2 mixture for 10–15 days. The gas phase was replaced every 4–5 days with the methane-air-CO_2 mixture.

Hydrogen evolving activity assay with whole cells

1 day old batch cultures of *Mc. capsulatus* (BATH), OD_{540}=0.25–0.50 were concentrated by centrifugation (10.000 rpm, 15 mins) and resuspended in 20 mM potassium phosphate buffer (pH 7.0) to give a final density of OD_{540}=1.0–2.0.2 ml of the resulting biomass were added, together with 1 ml 2 mM methyl viologen, to a special reaction vessel of 30 ml in volume. The reaction vessel enabled the replacement of the headspace with nitrogen, as well as, the prior addition of sodium dithionite into a separate compartment. After replacing the headspace with nitrogen, contact between sodium dithionite and the reaction mixture was established, resulting in the quick reduction of methyl viologen associated with the appearance of dark blue colour. This was considered as the zero time point of the reaction and incubation was carried out at 43°C. 500 µl of headspace were analysed after 20 min on a Hitachi 263–50 gas chromatograph. Operational parameters of the GC were as follows: 2 m long column of 1. 5 mm in diameter filled with 5 Å Molecular sieve. Injector, column and detector areas of the gas chromatograph were heated to 120°C. Nitrogen was used as the carrier at 50 ml/min flow rate.

All results represent the average of three separate assays.

Hydrogen uptake activity assay with whole cells

Fermenter grown cultures were concentrated and resuspended in 20 mM potassium phosphate buffer (pH 7.0) to give a final density of OD_{540}=1.0. Redox dye was added to the resulting cell suspension to give a final concentration of 0.2 mM for methylene blue (MB), 0.25 mM for methyl viologen (MV) and 0.4 mM for benzyl viologen (BV). 2 ml of cell suspension were added to anaerobic cuvettes of 5 ml total volume, stoppered with rubber Suba Seals. After replacing the headspace with nitrogen and 2 min of preincubation at 43°C, reactions were started by replacing a given percentage of the headspace with hydrogen. A Unicam UV/VIS UV2 spectrophotometer, equipped with a heated (to 42°C) multiple cell holder, was used to follow the reaction at 600 nm, in the case of methyl- and benzyl viologen and at 650 nm in case of methylene blue. The Vision/Rate and Spserv softwares were used to analyse the results. All results shown represent the average of at least three separate assays.

Hydrogen driven TCE degradation by whole cells

Mc. capsulatus (BATH) was grown in the fermenter in copper free NMS medium to a density of OD_{540}=5.5 to enable the expression of the sMMO. Bacteria were concentrated by centrifugation and resuspended in 20 mM potassium phosphate buffer (pH 7.0) to give a final density of OD_{540}=10.0. The reaction mixture (in a 25 ml conical flask) consisted of 4.0 ml 20 mM potassium phosphate buffer; 500 µl 1 mM TCE stock solution (aqueous) and 100 µl of 1 M sodium formate, if added (in which case, the volume of the phosphate buffer added was reduced correspondingly). 10 ml of the headspace was replaced by hydrogen or methane for hydrogen and methane driven TCE degradation assays, respectively. The reaction was started after 1 min of preincubation at 42°C by the addition of 500 µl cell suspension. The flasks were shaken at 200 rpm. 500 µl samples were withdrawn after 1 min and extracted with 500 µl n-pentane. 1 µl of the organic phase was then analysed on an SRI 8610C gas

chromatograph equipped with electron capture detector. 50 μM dibromoethane was used as an internal standard. Operational parameters of the gas chromatograph were: T_{column} = 50°C to 130°C in 3 mins; $T_{detector}$ = 200°C; $T_{injector}$ = 150°C; nitrogen carrier at 9 ml/min flow rate; 30 m x 0.53 mm capillary column filled with MXT-VDL (Restek Corp.).

Results and Discussion

Hydrogen evolution by Methylococcus capsulatus (BATH)

Hydrogen evolving activity was demonstrated from reduced methyl viologen at 108 ± 30 nmol hydrogen produced/min x mg dry cell activity. Hydrogen production at this rate by *Methylococcus capsulatus* (BATH) without prior induction indicated constitutive expression of the corresponding enzyme. In order to obtain further evidence for this hypothesis we carried out hydrogen uptake assays.

Hydrogen uptake assays

Even though there are several hydrogen utilisation assays described in the literature, hydrogen uptake prove very difficult to measure in methanotrophs. Part of the reason for this is that methanotrophs tend to store energy, mainly in the form of polyhydroxyalkanoates (PHA). PHA is metabolised during the assay and the derived reducing power is used to reduce the redox dye. Thus, a hydrogen free negative control is very likely to show some activity and this has to be subtracted from the result of the actual assay.

Assays with oxidised methyl viologen as the electron acceptor required a slight pre-reduction of methyl viologen. This was necessitated by the fact that the oxidised form of the redox dye was unable to cross the cytoplasmic membrane, as was found for *Thiocapsa roseopersicina* (BAGYINKA et al., 1983). The low standard redox potential of methyl viologen (–446 mV), however, enabled the production of hydrogen from the reduced portion of the dye. Production of hydrogen was demonstrated by gas chromatography. Consequently, subtracting the result of the negative control from that of the hydrogen containing assays resulted in a combination of hydrogen uptake and hydrogen production rates. This made reliable analysis of the results impossible.

Methylene blue was used as a redox dye because of its relatively high (+11 mV) standard redox potential, which does not allow the production of hydrogen from reduced methylene blue. However, due to this high standard redox potential, methylene blue is prone to be reduced by most redox systems of the investigated bacteria, resulting in a very high background activity. In many cases, this background activity was higher than the actual hydrogen uptake activity we tried to measure.

Benzyl viologen has a standard redox potential of -350 mV, slightly higher than that of methyl viologen. Fortunately, there was no need for pre-reduction of benzyl viologen for the hydrogen uptake assay. Moreover, the -350 mV standard redox potential was still too high for most of the redox systems of *Methylococcus capsulatus* (BATH), thus the problems encountered with methylene blue did not appear.

Hydrogen uptake rates from batch cultures of *Mc. capsulatus* (BATH) were not reproducible. Our hypothesis for this phenomenon is that exponentially growing batch cultures of methanotrophs use all the provided methane within 6 to 8 hours under the conditions applied. Thus, depending on the viability of the inoculum, an overnight batch culture of *Mc. capsulatus* (BATH) uses all the available methane 2 to 8 hours before harvesting and subsequent assaying. This may result in bacteria with highly variable physiological conditions (i.e. still very close to the exponential

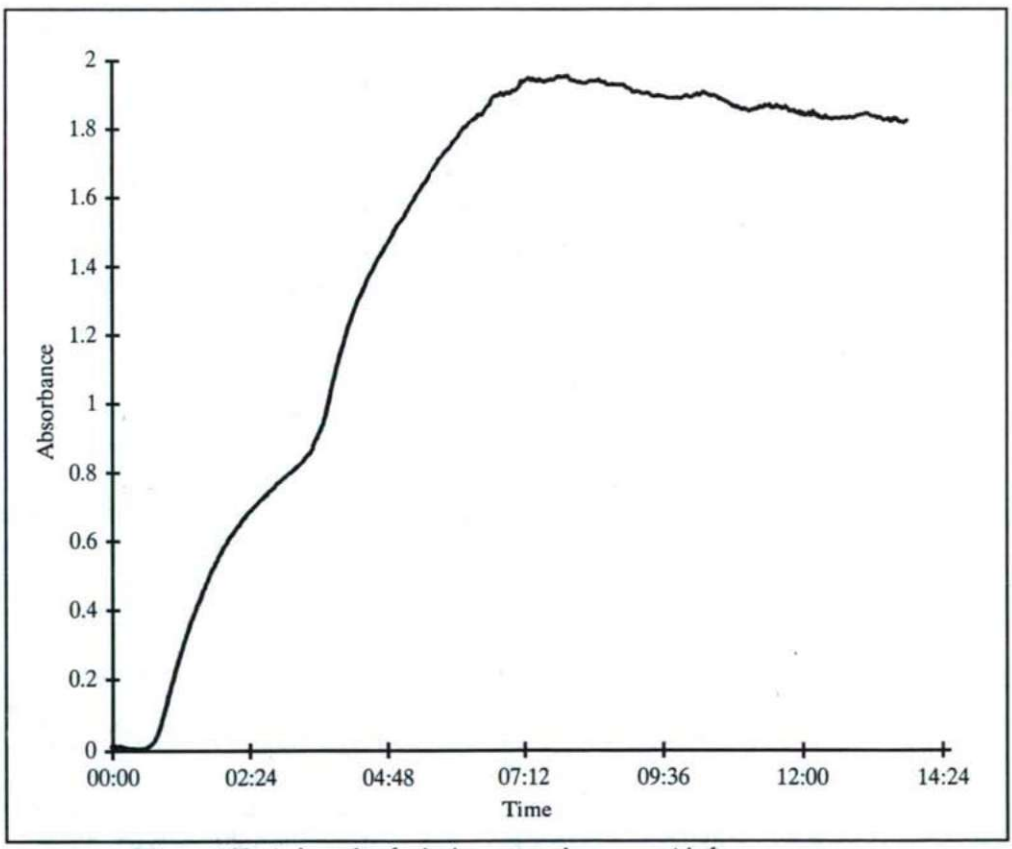

Figure 1 Typical result of a hydrogen uptake assay with fermenter grown *Methylococcus capsulatus* (Bath). The headspace contained 2% hydrogen.

phase or already in the late stationary phase of growth) between different assays. Continuous fermentation of methanotrophs reproducibly provided biomass in the exponential phase.

Hydrogen uptake activity showed a complex curvature (Fig. 1). The first part showed no dependence on the concentration of hydrogen in the headspace and activity values calculated from this curve were not reproducible. Thus all the activity data are derived from the second section of the hydrogen uptake curves.

Hydrogen uptake by Methylococcus capsulatus (BATH)

Hydrogen uptake measurements were carried out (applying the above described optimised assay with benzyl viologen on fermenter cultures of *Mc. capsulatus* (BATH)) under different concentrations of hydrogen in the headspace. Results (shown in Figure 2) indicate the constitutive expression of a hydrogenase with a K_M value for hydrogen of approximately 2%. Constitutive expression and high affinity for hydrogen are features which may be very useful for biotechnological applications of the hydrogen utilisation capability of *Mc. capsulatus* (BATH) (Fig. 2).

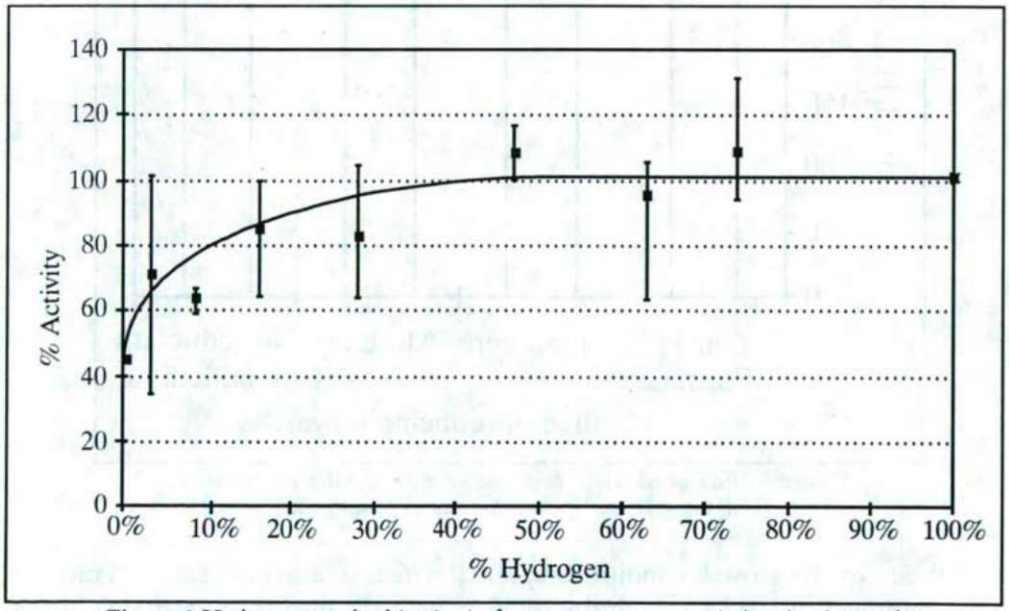

Figure 2 Hydrogen uptake kinetics in fermenter grown, non-induced culture of *Methylococcus capsulatus* (Bath).

Hydrogen driven TCE degradation by Methylococcus capsulatus (BATH)

To demonstrate the biotechnological potential of the hydrogen uptake activity of *Mc. capsulatus* (BATH), TCE degradation assays were carried out with various reducing sources (Fig. 3). There was a significant background activity (no reductant

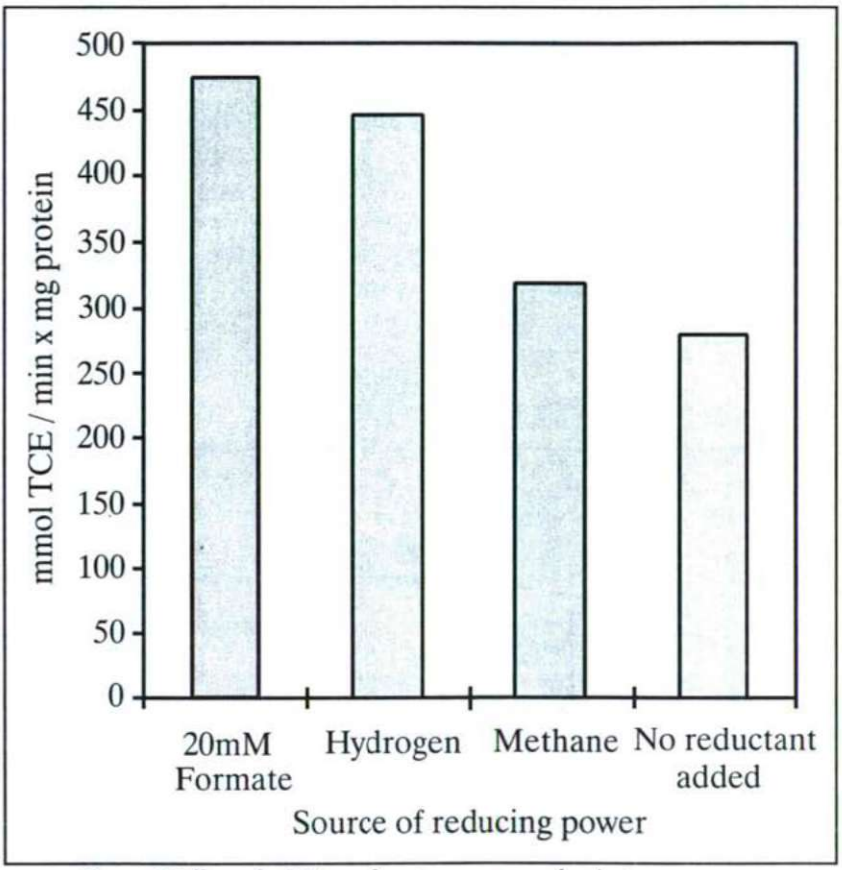

Figure 3 Effect of addition of various sources of reducing power on TCE degradation by *Methylococcus capsulatus* (Bath).

added) due to the growth conditions applied, which enabled the accumulation of high amounts of PHA. The addition of 50 % methane into the headspace did not increase the TCE degradation rate significantly. This is probably due to the competitive inhibition of TCE oxidation by methane, which was almost balanced by supplying reducing power via further oxidation of the methanol produced.

Addition of sodium formate did, however, cause a significant increase in TCE degradation, as found by many other authors. The effect of 50 % hydrogen in the headspace was comparable to that of sodium formate, with only a very slight difference. This result demonstrated the applicability of hydrogen uptake activity of *Mc. capsulatus* (BATH) in driving whole cell sMMO activity for the biodegradation of recalcitrant chlorinated hydrocarbons.

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